SEQUENTIAL MODIFICATION OF MEMBRANE CURRENTS WITH CLASSICAL CONDITIONING

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ABSTRACT Pavlovian conditioning of the nudibranch mollusc *Hermissenda crassicornis* was previously shown to produce long-lasting reduction of two K^+ currents measured across the Type B photoreceptor soma membrane (Alkon et al., 1982*a*; Alkon et al., 1985). Pavlovian conditioning of the rabbit was also shown to be followed by persistent K^+ current reduction (Disterhoft et al., 1986). Here we report the first evidence that Ca^{2+} currents can also be modified by conditioning. The amplitude of the currents rather than their voltage-dependence remains reduced at least 1–2 d after conditioning (but not control procedures). Conditioning-induced changes of both K^+ and Ca^{2+} currents increased as a function of training, the Ca^{2+} currents only changing substantially with $\geq$250 trials. The later changes of the Ca^{2+} current may function to limit the magnitude of excitability increases due to associative learning.

INTRODUCTION

A critical question in the neurobiology of learning and memory concerns the nature of the memory trace (Alkon, 1984; Alkon, 1987; De Lorenzo, 1980; Eccles, 1983; Hebb, 1949; Ito, 1982; Kandel and Schwartz, 1982; Krnjevic, 1986). What are the biological means of actually recording for future recall that two or more stimuli have repeatedly occurred with a fixed temporal relationship? Pavlovian conditioning provides an analyzable behavioral measure that such a temporal relationship has actually been learned by an animal. Previously we identified the first example of a biological memory trace which persists at least 2–3 d after conditioning. Persistent reduction of an early K^+ current, I_A, and a late K^+ current, not pharmacologically separated from other K^+ currents, but most likely $I_{Ca^{2+}-K^+}$, were causally implicated in the retention of Pavlovian conditioning of the nudibranch mollusc, *Hermissenda crassicornis* (Alkon, et al., a, b 1982; Alkon et al., 1985). Recently a striking parallel to this biophysical trace was found in the mammalian brain. Conditioning-specific reduction of the after-hyperpolarization (the AHP, a measure of a Ca^{2+}-dependent K^+ current) was measured for CA1 neurons within rabbit hippocampal slices (Coulter et al., 1986; Disterhoft et al., 1986).

In addition to the voltage-sensitive outward K^+ currents measured across the Type B soma membrane, a voltage-sensitive inward Ca^{2+} current has also been described (Alkon et al., 1984). This inward current, most apparent when Ba^{2+} was substituted for extracellular Ca^{2+}, was only significantly activated at potentials ≥ -10 mV (absolute), was blocked by 10 mM Cd^{2+} in the bathing medium, and showed no appreciable inactivation (Alkon et al., 1984). We report here that with prolonged training (but not with moderate numbers of training trials) this current is clearly reduced when measured 1–2 d after classical conditioning (but not control training procedures). In contrast to the $I_{Ca^{2+}}$ dependence on training, $I_{Ca^{2+}-K^+}$ is substantially reduced by $\leq$100 training trials and diminishes very gradually with further training thereafter. As discussed below, the overall effect of these conditioning-specific changes may be to impose some limit on the degree to which a cell's excitability can be enhanced with experience.

METHODS

Behavior and Maintenance

All animals were obtained from Sea Life Supply, Sand City, CA, and maintained at 11–13°C in 0.22 mm-filtered seawater in a refrigerated aquarium (Dayno Manufacturing Co.). Two fluorescent bright sticks (Sylvania Corp.) provided illumination in a cycle of 12 h light, 12 h dark. They were fed daily on ~60 mg of green crab (*Carcinus maenas*) viscera. Animals were trained (Alkon et al., 1985) on a daily schedule of 100 pairings of light and rotation (paired or conditioned group) or randomly associated light and rotation (random group). A third group that received no training (naive group) was used as an additional control. Before each training session, the animal's reactivity to the light source (#6500 cadmium blue light spectral line 1 amplifier; Oriel Corp.) was assessed as a latency to move 1 cm in a lucite center-start tube with an inside diameter of 12.8 mm, filled with artificial sea water (ASW). The intensity of the light source at the center of a spot 15 cm distant was 3.5×10^3 ergs/cm^2-s. This latency to move 1 cm, really a start latency, was

considered to reflect more directly inhibition of a movement due to foot contraction. Light-elicited foot contraction is a new response produced by classical conditioning of *Hermissenda* with repeated light-rotation pairings (Lederhendler et al., 1986). The center-start tubes employed here differ from previous ones used in this laboratory in that they allow a bidirectional movement of the animal, i.e., towards or away from the light. Here, therefore, only the latency to move 1 cm in either direction after light onset was used as an index of photoresponsivity. However, the latency measure used here was found to be highly correlated ($p < 0.005$) with the latency measure used previously involving directed movement toward a light source. Animals (~5%) which took longer than 10 min to move on initial testing were discarded. After 4 d of training, and a total of 400 pairings, all animals (unless otherwise specified) were submitted to a final testing and voltage-clamp recording on the fifth day.

Voltage Clamp

Medial Type B photoreceptor somata were isolated by axotomy as previously described (Alkon et al., 1984; Alkon and Sakakibara, 1985). The cells were maintained at 20–22°C in ASW (NaCl: 430; KCl: 10 mM; $MgCl_2$: 50 mM; $CaCl_2$: 10 mM; Tris: 10 mM) and impaled with two glass microelectrodes with resistance 10–15 MΩhms and filled with 3 M KCl. Impalement was achieved after enzymatic digestion of the surrounding connective tissue with protease (Type VI, Sigma Chemical Co., St. Louis, MO). No cells were accepted for further recording if: (*a*) there was more than a 2 mV difference in resting potential recorded by the two microelectrodes; (*b*) the light responses recorded by the two microelectrodes were not identical; (*c*) the resting membrane potential did not steadily become more negative with progressive dark adaptation; (*d*) the input resistance and/or the light response became smaller; or (*e*) more than 20 min elapsed between end of enzymatic treatment and impalement.

The extracellular bathing medium was then replaced with ASW containing 300 mM KCl, 50 mM $MgCl_2$, 35 mM NaCl, 10 mM $CaCl_2$, 10 mM Tris, 100 mM TEA, and 5 mM 4-aminopyridine. After exchanging the chamber volume (containing ~2 cc) fifteen times, the Type B cell soma membrane potential was voltage clamped to −60 mV. Voltage-clamp techniques were as previously described (Alkon et al., 1984; Alkon et al., 1982*a*; Alkon and Sakakibara, 1985; Alkon et al., 1985). By means of a computer subroutine, the exact reversal potential for each cell in this elevated K_o^+ was determined by two separate methods which showed exact agreement. By the first method (Fig. 1, *A* and *B*) that potential at which there was no inward current with a slow rise to maximum amplitude but only an inward current with a rapid rise to maximum during the command step was considered to be the reversal potential. During command steps to >0 mV, the rise to maximum became more complex and for steps ≥10 mV, an outward current with a slow rise to maximum appeared. By the second method (Fig. 1, *C* and *D*) that potential at which the tail current after the command step disappeared was considered to be the reversal potential. Reversal potentials obtained in this way for each cell were identical invariably within ±2 mV of 0 mV (see previous measurements in Alkon et al., 1984). Command pulses to the measured reversal potential for each cell elicited an inward sustained current, previously shown to be Ca^{2+} (Alkon et al., 1984). Thus, as previously reported (Alkon et al., 1984), the inward voltage-dependent Ca^{2+} current, $I_{Ca^{2+}}$, could be measured in isolation from contaminating $I_{Ca^{2+}-K^+}$ with command depolarizing steps to 0 mV, at which there was not net K^+ flux. This method of measuring $I_{Ca^{2+}}$ was previously found (Alkon et al., 1984; Alkon and Sakakibara, 1985) to cause minimal deterioration of the Type B cells as indicated by stability of leak current, input resistance, holding current, and duration of recording. By contrast, reduction or elimination of K^+ currents by injection of cesium ions and/or substitution of external Ca^{2+} with Ba^{2+} usually allowed only brief periods of cell viability. All current measurements were corrected for the nonvoltage-dependent or leak current. For leak current measurements, 100 ms pulses of opposite polarity but equal amplitude at each depolarizing level were used. The currents obtained with these hyperpolarizing pulses showed a linear relationship between current and voltage. A measure of $I_{Ca^{2+}-K^+}$, shown in an earlier study (Alkon et al., 1984) to be resistant to 100 mM TEA, was obtained as a tail current following each command depolarization. Since quantitative $I_{Ca^{2+}-K^+}$ measurements required at least 30 s between each command depolarization, tail current values were available for fewer cells than those cells for which $I_{Ca^{2+}}$ could be measured. The PDP 11/34 computer (Digital Equipment Corp., Maynard, MA) which was used generated 1 s command pulses, and sampled the current recording at 500 μs.

RESULTS

Behavior

One or two days after training, *Hermissenda* exposed to training with paired light and rotation (the conditioned group) showed a significantly longer (4.71 s ± 3.1) latency to move in response to light than those animals exposed to randomly occurring light and rotation (the random group: 2.8 s ± 1.1; $t = 2.09$, $p < 0.002$, one-tail). Conditioned *Hermissenda* also showed a significantly longer latency than animals exposed to no light or rotation (the naive group; 2.51 s ± 2.1; $t = 3.14$, $p < 0.01$, one-tail).

Voltage Recordings

Impalements of Type B cell somata were made 1–2 d after training for the three groups using a double-blind procedure. Resting membrane potentials were not significantly different between groups (Conditioned: 35.2 ± 7.8 mV, $N = 10$; Random: 35.9 ± 9.9 mV, $N = 11$; naive: 36.3 ± 8.8 mV, $N = 15$). Resting membrane potential for cells of all three groups approached 50 mV during progressive dark adaptation (for 5–10 min).

Calcium Currents

In 300 mM K_o^+ and potassium channel blockers (100 mM tetraethylammonium (TEA) and 5 mM 4-aminopyridine [4-AP] a sustained, voltage-dependent inward current was maximally activated at command steps between −10 and +10 mV. This current was almost totally blocked 7 min after the addition of 10 mM Cd in the extracellular bathing medium (Fig. 2). A tail current, carried by the reversed calcium-dependent potassium current $I_{Ca^{2+}-K^+}$, was also reduced, but there was no significant change in the light-induced inward sodium current (Fig. 2).

Progressive elevation of extracellular K^+ to 400 mM in the bathing medium changed the equilibrium potential for K^+ flux from −40 to −20 to 0 and finally to +20 mV. Current-voltage plots were obtained by step commands to each one of these potential levels (for each new K^+ equilibrium potential). In a subpopulation of six animals which had experienced 250 trails of paired light and rotation over a 3–4 d period (which causes clear behavior differences) (Alkon, 1987) the shape of the current-voltage relation was not altered, although the conditioned cells showed more positive values along the current axis (Fig. 3) as compared with naive (5) animals. For these two subpop-

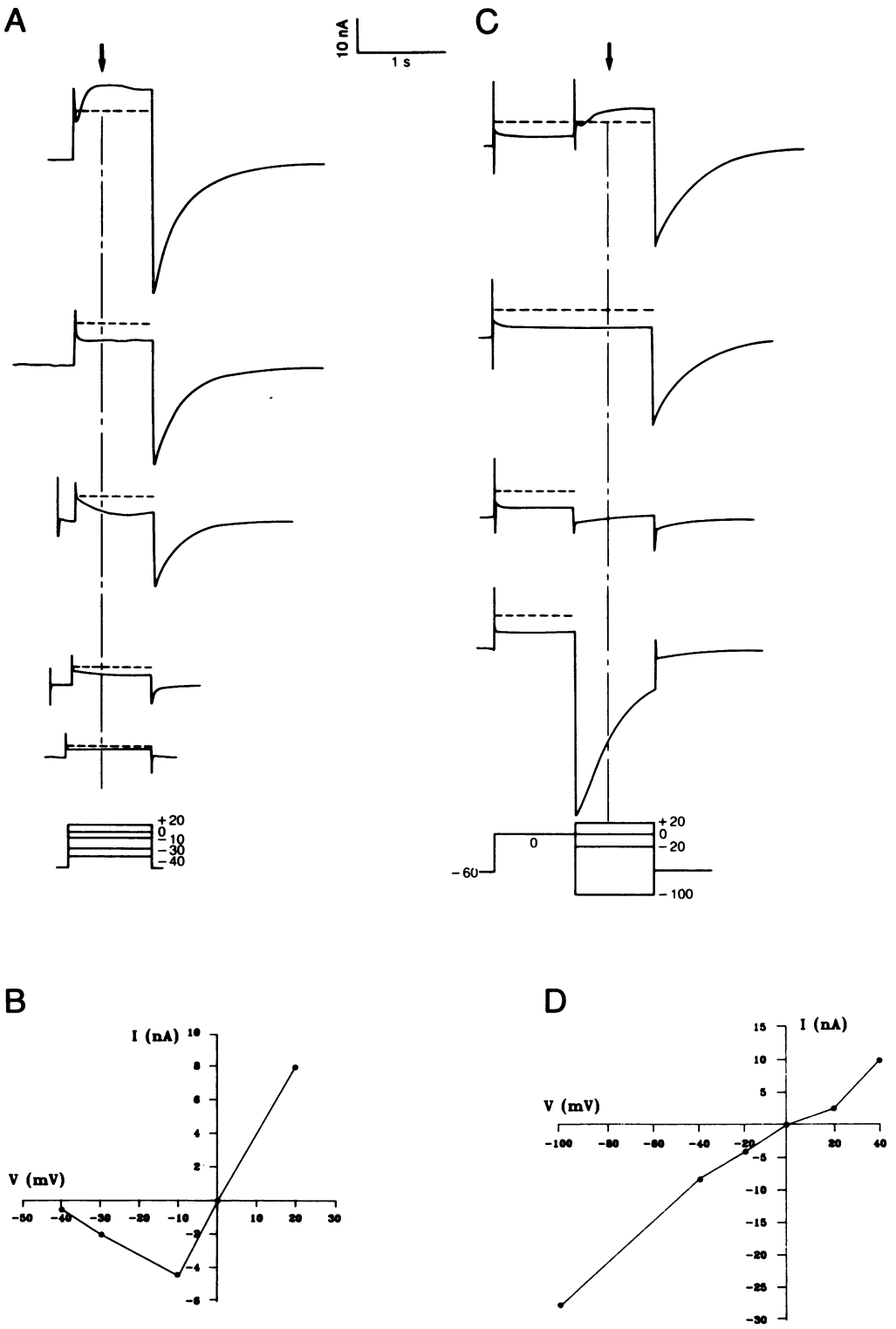

FIGURE 1 Voltage clamp recordings from a medial type B photoreceptor in an extracellular solution containing high potassium (300 mM) and potassium channel blockers 4 AP and TEA. (*A*) Inward currents with a delayed rise to maximum are elicited by steps <0 mV. At 0 mV a sustained inward with a rapid rise to maximum is apparent. For steps >10 mV outward currents with a delayed rise to maximum are elicited. Vertical dashed line indicates isochronal measurements plotted in *B*. (*B*) I-V plot for currents obtained in *A*. Note the reversal potential at 0 mV absolute. (*C*) Standard depolarizing prepulses to 0 mV are followed (<10 mS) by test pulses. During test pulses <0 mV, the tail currents are inward and for test pulses >0 mV, the tail currents are outward. Note the sustained inward current during both the prepulse and test pulse at 0 mV. Vertical dashed line indicates isochronal measurements plotted in *D*. (*D*) I-V plots for tail currents obtained in *B*. Note the reversal potential of 0 mV absolute. Horizontal dashed lines indicate level of the leak current.

ulations the peak inward Ca^{2+} current, $I_{Ca^{2+}}$, was significantly smaller ($p < 0.01$ by a two-tailed t-test) for the conditioned as compared with the naive cells.

Since the amplitude of the $I_{Ca^{2+}}$ currents rather than their voltage-dependence appeared to be altered by conditioning, we examined three additional populations for maximal $I_{Ca^{2+}}$ currents. For conditioned, randomized control and naive animals, the amplitude of a voltage-dependent inward Ca^{2+} current, $I_{Ca^{2+}}$, was measured, after at least 10 min of dark adaptation, in 300 mM K_o^+ in the presence of 5 mM 4-AP (to block I_A) and 100 mM TEA (to block the delayed rectifier, I_K). The equilibrium potential for K^+ currents in 300 mM K_o^+ was ~0 mV (absolute). At this potential $I_{Ca^{2+}}$ is maximally activated and it was

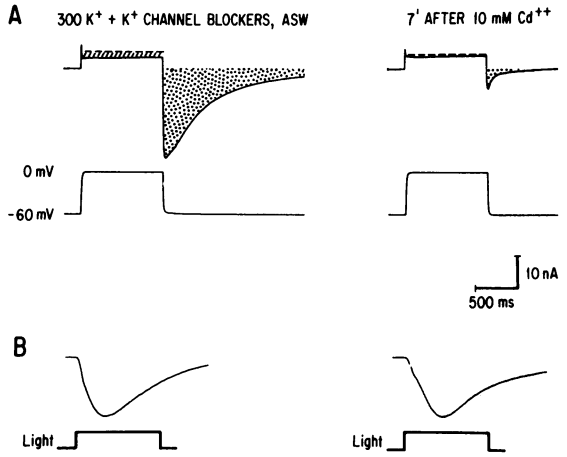

FIGURE 2 Calcium currents in *Hermissenda* medial Type B photoreceptors. (*A*) Two microelectrode voltage-clamp recordings of medial Type B photoreceptor membrane current in an extracellular solution containing high potassium (300 mM) and potassium channel blockers (100 mM TEA and 5 mM 4-AP) in artificial seawater. Dashed area shows an inward sustained current after leak correction. The tail current is not blocked by the previous K^+ channel blockers and has a reversal potential of approx. O mV. Seven minutes after the addition of 10 mM Cd, both inward and tail currents are blocked (*records on right*). (*B*) Light-induced inward current carried by Na^+, elicited by a 2 s light step (10^4 ergs/cm^{2-} s) is not blocked by Cd (*on right*). Dashed lines indicate level of nonvoltage-dependent or leak current.

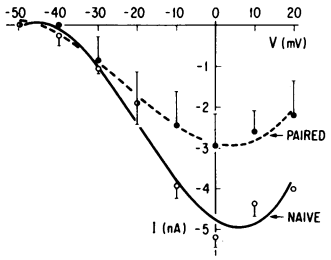

FIGURE 3 Current-voltage relations of $I_{Ca^{2+}}$. To obtain $I_{Ca^{2+}}$ at different voltage levels, extracellular K^+ was progressively elevated, with resulting shifts of the equilibrium potential for K^+ flux from −40 to −20 to 0 and to +20 mV. Value curves were means obtained from conditioned or paired ($N = 6$) and that while conditioning alters the peak current amplitudes, it does not alter their voltage-dependence.

possible to observe conditioning-specific reduction of I_{Ca}^{2+} (Fig. 3, 4, Table 1). There were, however, no between-group differences in the non-voltage-dependent or "leak" current (cf. Table 1).

Calcium-dependent K^+ currents

The amplitude of the outward Ca^{2+}-dependent K^+ current, $I_{Ca^{2+}-K^+}$, could also be measured as an inward tail current 10 ms or longer after the command depolarization. Since no significant $I_{Ca^{2+}}$, I_{Na}^+, I_A, or I_K^+ occurs at −60 mV (with 4-AP and TEA present), $I_{Ca^{2+}-K^+}$ was measured in relative isolation of other currents. Under these conditions there was an unequivocal conditioning-specific reduction of $I_{Ca^{2+}-K^+}$ (Fig 4, Table I). This latter observation is consistent with a previous report (Alkon et al., 1985) demonstrating a conditioning-specific reduction of a late outward K^+ current, which, based on the pharmacologic studies, was interpreted to be largely $I_{Ca^{2+}-K^+}$.

Changes of Currents as a Function of Training

The Ca^{2+} current and K^+ tail current were measured in animals which received different numbers of training sessions (0 trails; $N = 8$, 50 trails: $N = 5$; 100 trials: $N = 7$; 200 trials: $N = 9$; 300 trails: $N = 8$; 400 trials: $N = 6$). For 100 or less presentations of paired light and rotation the K^+ current was 40% reduced ($p < 0.01$) as compared with control or naive levels (Fig. 5), while the Ca^{2+} and leak currents were not significantly affected. With progressively more training, however, the Ca^{2+} currents (but not the leak current) slowly became smaller, ultimately showing a highly significant reduction ($p < 0.002$) of ~50% with 400 trials (Figs. 4, 5, and Table I). With more than 100 stimulus pairs the K^+ currents also showed some further gradual reduction.

TABLE I
VOLTAGE-DEPENDENT INWARD CA^{2+} CURRENTS AND CA^{2+}-K^+ TAIL CURRENTS OF TYPE B MEDIAL CELLS, RECORDED UNDER THE SAME CONDITIONS AS IN FIG. 1

	ICa^{2} + (nA)	ICa^{2+}-K^+	Leak current (nA)
	$x \pm$ SD	$x \pm$ SD	$x \pm$ SD
(C) Conditioned	(N = 10) − 1.83 ± 0.64	(N = 7) 5.57 ± 3.2	(N = 10) 9.75 ± 1.56
(R) Random	(N = 11) − 4.49 ± 1.6	(N = 5) 10.0 ± 2.5	(N = 11) 9.22 ± 2.13
(N) Naive	(N = 15) − 4.18 ± 1.2	(N = 9) 10.7 ± 5.0	(N = 15) 9.33 ± 1.82
C vs. R	t^* = 5.07, $P < 0.0002$	$T^‡$ = −2.71, $P < .01$	T^* = 0.57, NS
C vs. N	t^* = 5.25, $P < 0.00001$	t^* = −2.47, $P < .01$	T^* = −0.63, NS
R vs. N	t^* = −0.53, NS	$t^‡$ = 0.33, NS	T^* = 0.13, NS

*Two-tailed t-test.
‡One-tailed t-test.
Values are given as means (X) and standard deviations (SD) in the three groups. NS means nonsignificant. The N values represent the number of animals in each group. Only one cell per animal was accepted for the comparisons. No significant difference (by t-tests) was observed in leak currents between groups: conditioned: 9.75 ± 1.56; random: 9.22 ± 2.13; naive: 9.33 + 1.82.

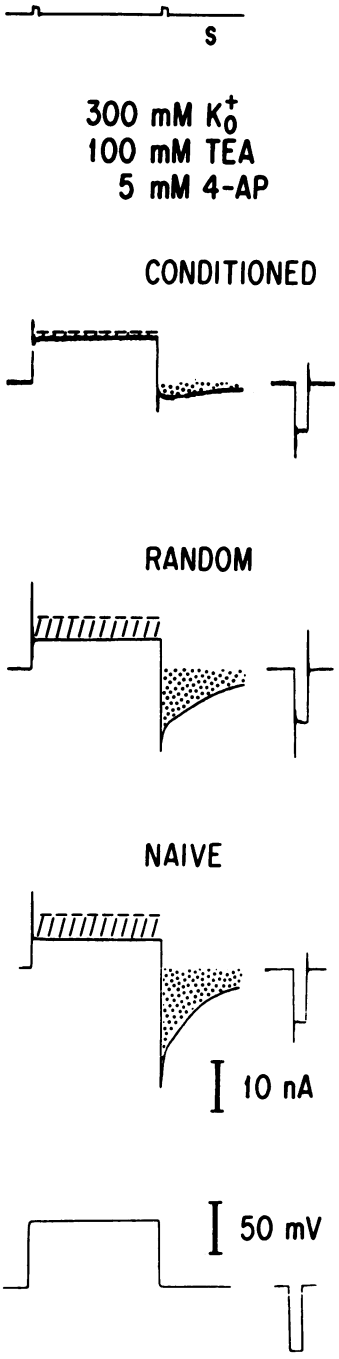

FIGURE 4 Voltage-dependent inward Ca^{2+} currents ($I_{Ca^{2+}}$) from conditioned, randomized control and naive animals. Two microelectrode voltage-clamp recordings of medial Type B photoreceptor membrane currents in an extracellular solution containing high potassium (300 mM) and potassium channel blockers (100 mM TEA and 5 mM 4-AP) in artificial seawater (ASW). Command steps to O mV (absolute) elicited a significantly smaller $I_{Ca^{2+}}$ (*dashed areas*) in the conditioned animals. Similar changes in the tail currents, due to a calcium-dependent K^+ flux, are also illustrated (as shaded areas following the command steps). The dashed line represents the level of the voltage-independent or leak current.

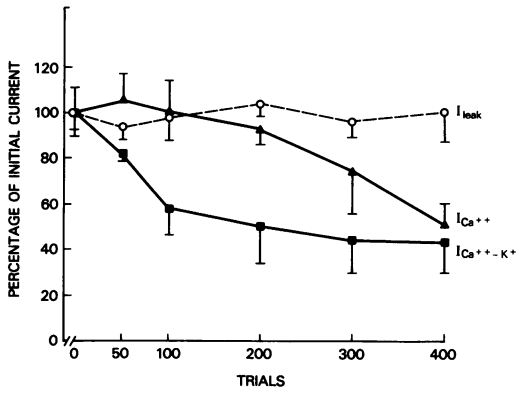

FIGURE 5 Membrane currents change sequentially with increasing numbers of training trials. $I_{Ca^{2+}}$ (▲), $I_{Ca^{2+}-K^+}$ tail current (■), and leak (O) were obtained with the same recording protocol as for Fig. 1. Values shown are means ±SEM of the percentage changes in current amplitudes of animals with 50 (N = 5), 100 (N = 7), 200 (N = 9), 300 (N = 8), and 400 (N = 6) training trials, compared with naive (N = 8) untrained control group. Note that there was no significant change of leak currents as a function of training.

DISCUSSION

Neuroanatomic and electrophysiologic data have demonstrated that visual information from the *Hermissenda* eye is distributed along (at least) three different neuronal pathways. One of these concerns the turning movements of the animal, another concerns contraction of its undersurface or foot (Lederhendler et al., 1986). Therefore, the animal's response to a visual stimulus (e.g., a light gradient) can be complex, involving the integration of different visually-elicited behavioral components. In the past, the least specific assay of the *Hermissenda* photoresponse was provided by the time required for the animal to move toward a light source. Another measure was provided by the time required for the animal to turn at a light-dark border. Perhaps the measure with potentially the most direct relevance to contraction of the muscular foot was provided by time-lapse photographic images of the foot under various conditions of visual and vestibular stimulation. With this measure, Lederhendler et al. (1986) showed that light-rotation pairings produced an actual transfer of rotation-elicited foot contraction to the conditioned stimulus, light. As a result of conditioning, light elicited an entirely new response, foot contraction, which resembled the unconditioned response. Furthermore, the extent of foot contraction was inversely related to the animal's movement velocity.

Here, we used as a crude measure of this foot contraction (and, therefore, indirectly of its movement velocity) the time required for the animal to move at all, i.e., in any direction. This was thought reasonable since foot contraction should reduce movement of the animal in any direction. It was not entirely surprising, therefore, that the conditioned group showed a significantly longer latency to move 1 cm in any direction in response to light. Nor was it unexpected that this longer latency was highly correlated with the latency measure used in previous studies.

When Ca^{2+} was previously iontophoresed through one microelectrode into a Type B soma held under voltage clamp at −60 mV via two additional microelectrodes, the K^+ currents, I_A and $I_{Ca^{2+}-K^+}$, were reduced in the absence of $I_{Ca^{2+}}$ reduction (Alkon and Sakakibara, 1984). Similarly, with paired command depolarizing steps, $I_{Ca^{2+}-K^+}$ underwent marked inactivation in the absence of $I_{Ca^{2+}}$ inactivation (Alkon et al., 1984; Alkon and Sakakibara, 1985). When, however, external Ca^{2+} was sufficiently elevated (e.g., from 10 to 100 mM), $I_{Ca^{2+}}$ showed inactivation with the paired pulse regimen. These results suggested that $I_{Ca^{2+}}$

can be inactivated by elevation of cytosolic Ca^{2+} but only at levels greater than those necessary to inactivate I_A and $I_{Ca^{2+}-K^+}$. These in vitro data may help explain our present finding that sufficient conditioning in vivo is accompanied by prolonged reduction of $I_{Ca^{2+}}$. The in vitro difference in the sensitivity of $I_{Ca^{2+}}$ and $I_{Ca^{2+}-K^+}$ to Ca^{2+} elevation was paralleled by the observed in vivo difference in the sensitivity of these currents to training experience.

It is interesting that, under conditions when I_A and $I_{Ca^{2+}-K^+}$ might undergo marked inactivation, such as prolonged light-adaptation, (Alkon and Sakakibara, 1985; Alkon et al., 1982*b*) $I_{Ca^{2+}}$ is sustained and does not inactivate. Under light-adapted conditions, therefore, a conditioning-specific reduction of $I_{Ca^{2+}}$ might make the cell less excitable and account for different response characteristics of the cell and the animal to visual stimuli. We might also speculate that conditioning-induced modification of $I_{Ca^{2+}}$ could serve as a saturation mechanism whereby the cell excitability is not altered too dramatically in one direction. Reduced $I_{Ca^{2+}}$ also would itself decrease subsequent Ca^{2+} elevation and thereby limit additional modification of ionic currents. The results indicate that acquisition of a learned association involves a sequence of biophysical modifications. Such a sequence is reminiscent of the progressive modification of membrane channels found for developing cells and as such might reflect more general principles of cellular transformation (Cowan, 1981).

Finally, mention should be made of the recent findings that the current required for eliciting Ca^{2+} spikes greatly increases for CA1 neurons in hippocampal slices isolated from conditioned (but not control) rabbits (Coulter, 1986). The latter findings suggests a possible further parallel to the *Hermissenda* results: namely, that for CA1 cells $I_{Ca^{2+}}$ as well as $I_{Ca^{2+}-K^+}$ are reduced on days after Pavlovian conditioning.

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